Targeting *Mungbean Yellow Mosaic Virus* (MYMV) using RNAi-based nanoformulation for sustained protection

Synopsis

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INTRODUCTION

Vigna radiata,(L.) generally known as mungbean, is a legume crop which holds third position in the category of self-pollinated plants (Kumar *et al*., 2014). This plant possesses small genome with 22 chromosomes as 2n number (Parida *et al*., 1990; Kang *et al*., 2014). Whitefly (*Bemisia tabaci* Gennadius), being the vector host, causes the transmission of *Mungbean Yellow Mosaic Virus*. It causes Yellow Mosaic disease and hence largely affects the crop (Karthikeyan *et al*., 2014). Infected plant shows noticeable symptoms on leaves like yellow- coloured spots dispersed all through the surface, which gradually cause yellowing of whole leaves. Consequently, it causes decrement in photosynthetic capacity and sooner or later results in drying and wilting of leaves (Malathi *et al*., 2008). At first, *Mungbean Yellow Mosaic* viral particles was detected by Thongmeearkom *et al*., 1981 and firstly purified by Honda *et al*., 1983 in cells isolated from leaves.

Originally, whitefly is an Indian polyphagous pest i.e. it feeds on various species of crops (Fishpool *et al*., 1994). Virus enters in vector host when it is feeding on phloem sap of the plant. Through the haemolymph circulation, viral particles enter the salivary glands of the pest and at the time of subsequent feeding, it is transmitted to fresh plants (Fiallo-Olive *et al*, 2020). It is to be noticed that age and gender of the pests are important parameters that need to be considered while studying virus transmission efficiency (Czosnek *et al*., 2002).

Out of several known infections in the plants by several pathogenic microorganisms, viruses contribute majorly to crop loss throughout the globe (Marwal *et al*., 2020). Due to the lack of an efficient approach and effective procedures to oversight viral infection, they impose a great risk in damaging the wide range of agricultural crops and thus cause economic losses too (Cai *et al*., 2019; Singh *et al*., 2019). Viruses are obligate intracellular parasites that cannot survive or proliferate without any host. They spread and multiply using the molecular machinery of the host and consequently, contaminate the whole plant and gradually the whole farm (Cai *et al*., 2019; Llave *et al*., 2016). Viral infections can cause mosaic, mottling, wrinkled leaves, and underdeveloped fruits, which consequently affect physiological functions and cause impairments, like necrosis, decelerated plant growth, and even death, therefore pose a huge threat to agriculture production throughout the planet (Yang *et al*.,

2018). The sole practice to control or rule out the virus is to detect the infected plant or the host for viruses and remove it from the rest of the plants as early as possible (Quatrini *et al*., 2018).

RNA interference (RNAi), also termed as RNA silencing, is an endogenous cellular mechanism that is found in all higher eukaryotes (Voloudakis *et al*., 2014). RNAi offers a great therapeutic procedure for disease management (Ballarín-González *et al*., 2012). It is a sequence-specific, post-transcriptional gene silencing mechanism that is induced by double-stranded (ds) RNA and it serves as a protective mechanism for cells against the foreign nucleic acids like viruses and transposons that have infected the cell (Konakalla *et al*., 2016). dsRNA molecules homologous to a particular invasive mRNA, initiate silencing upon interaction with the RNA-induced silencing complex (RISC) and thus through a proper cascade pathway and subsequent involvement of various proteins and enzymatic actions, it results in sequence-specific degradation (Konakalla *et al*, 2016; Ballarín-González *et al*., 2012). Since, it is a defensive mechanism that provides protection to plants, it is been exploited in plant biotechnology to promote disease resistance against pathogenic microorganisms.

The methods employing transgenic techniques and the use of virus-derived vectors are tedious and possess lower efficiency that is species-dependent. They have a bit adverse effect to human health (Jiang *et al*., 2014) and also impose negative effects ecologically, like, heterologous viral encapsidation, and genetic flow between organisms (Konakalla *et al*, 2016; Bartlett *et al*., 2006). Because of these few reasons, RNAi is utilised as it is a more biocompatible, safer, and efficient approach for non-transgenic antiviral resistance (Jiang *et al*., 2014; Bartlett *et al*., 2006). As compared to the transgenic approach, RNAi is preferred due to certain advantages; such as, it is a less time-consuming and tiresome process, moreover lesser cost-intensive. Besides all these, this strategy checks the use of pesticides and therefore environmentally friendly (Konakalla *et al*, 2016).

In recent times, nanobiotechnology tools and techniques have evolved epidemically in terms of their applications. Nanoparticles mediated RNAi technology is being refined to use it as an alternative to pest management strategy (Das *et al*., 2015). These technologies have attempted to offer hopeful alternatives to minimise the threat to plant good health. Lately, nanoparticles are being interacted with the disease management mechanisms to develop a potent agronomical tool for plant protection and its health (Elmer *et al*., 2018; Cai *et al*., 2018; Fang *et al*., 2015). Due to their nanosized dimensions, they are efficiently

adopted as delivery vehicles to transport genetic material in the plant cell. Few studies suggest that nanoparticles are effective in eliminating the virus particles and further they can activate the plant immune system to resist further viral infection. Most semimetals and metallic oxide nanoparticles are used to target the pathogens as bactericides, fungicides, and nano pesticides as well, while few of them serve as nano fertilizers that reinforce the plant's health and productivity (Cai *et al*., 2019).

Being a defense system, plants utilise RNAi to provide autoimmunity against foreign viruses. To overcome the RNAi of the host, viruses encode 'silencing suppressors' (VSRs) as their counter defence mechanism. For instance, the 2b protein is one of the proteins that is encoded by the *Cucumber mosaic virus* (CMV) to prevents the mobilisation (Konakalla *et al*., 2016) of viral RNA silencing signal to distal tissues (Guo *et al*., 2002). This signal suppresses RNAi and therefore allows CMV to infect the plant host. Various other examples include The P19 protein of *Carnation Italian ringspot virus* (CIRV) and helper component-protease (HC-Pro) of some potyviruses bind to 21 nucleotide viral small interfering RNAs (vsiRNAs) and thus obstruct the spread of RNA silencing, whereas P38 protein of *Turnip crinkle virus* promotes virus propagation by binding vsiRNAs of 21 and 22 nucleotide in size which are produced by DCL4 and DCL2, respectively (Lakatos *et al*., 2006; Ding *et al*., 2007). Few cellular proteins of host and viroid RNA motifs facilitate Viroid intercellular transport. To detect small RNAs, like mature miRNAs, the stem-loop PCR technique has been used (Konakalla *et al*, 2016).

Nanocarriers ensure the enhanced stability and sustained release of the nucleotide. At the present time, the details regarding the mechanisms of the intercellular and distant trafficking or mode of entry of siRNAs as well as long dsRNA molecules in plants are still restricted (Mitter *et al*., 2017). Development of stable construction and cost-effective mass production of dsRNA and development of the economic delivery system is the major challenge to use this technique on a larger scale boundlessly (Das *et al*., 2015). Furthermore, the stability of dsRNA during the process defines the success of this technique. For prolonged protection, it must remain on the plant surface for a longer period and must be able to cope up with the natural environmental conditions like rain, sunlight (Mitter *et al.*, 2017).

In the current study, biodegradable PLGA NPs will be used for siRNA delivery which breaks down into non-toxic, biocompatible residues i.e., H_2O and CO_2 (Rezvantalab *et al*., 2018). NPs will be prepared using emulsion-based techniques which is a rapid approach involving low-cost equipment. Furthermore,

NPs will be retrieved in powder form which will in turn enhance the stability and durability of NPs and would also reduce the storing capacity and hence the transport cost (Operti *et al*., 2021).

The relevance of this work is to provide a new prospect in the field of plant disease management utilising biocompatible nanoformulation as vehicle for siRNA delivery and consequently bring about reduction in the use of harmful chemicals.

OBJECTIVES

The basic aim of the research is to make an attempt to develop an effective and eco-friendly technique that can be used on a larger scale by the farmers worldwide so as to minimise the crop as well as the economic losses caused by viral infections.

The main objectives will be:

- Identification and characterization of *Mungbean Yellow Mosaic virus*.
- Designing and preparation of precursor siRNA against target gene.
- Development of cationic nanoparticle and conjugation of precursor siRNA to nanoparticles.
- To study the efficacy of nanoformulation against plant virus.

LITERATURE REVIEW

Each year, more than 700 species of crop are infected by virus and causes nearly about 60 billion US $ economic loss according to a report by Das and colleagues 2015. The infection obviously results in the serious reduction as well as the devaluation of crop and thus affects the food availability across the globe. Besides the various aspects like rapid transformation of virus into a new strain, noxious effects of insecticides and pesticides, poor practices of farmers on field; Unavailability of any viricide is the major challenge that has to be faced in controlling the viral infections (Mitter *et al*., 2017).

At first, Tenllado and Diaz-Ruiz confirmed gene silencing by administrating dsRNA by upon plants against *Pepper mild mottle virus* (PMMoV), *Alfalfa mosaic virus* (AMV) and *Tobacco etch virus* (TEV). dsRNA homologous to the respective target gene was co-inoculated with the virus and reduced formation of virus-induced lesions were observed on the administrated leaves. These examinations were reciprocated with different hosts and other viruses, reduced or absence of any infection was recorded in the co-inoculated leaves (Tenllado *et al*., 2001). With the measured application, nanoparticles are

noticeably efficient in inhibiting the infections caused by pathogenic microorganisms and thus boost up the plant development (Sathiyabama *et al*., 2018; Kumaraswamy *et al*., 2018).

Cai and colleagues 2019 revealed that the zinc oxide (ZnONPs) and silica nanoparticles (SiO_2NPs) possess antiviral characteristics and therefore used against *Tobacco mosaic virus* (TMV) infection. TMV particles aggregated when they were pretreated with either ZnONPs or SiO_2NPs for 2 hours. When this mixture was inoculated onto the tobacco leaves, diminished viral infection was observed for 2 days after application. But 7 days after application, it was incapable to combat replication and hence viral infection. Consequently, due to treatment with these nanoparticles, systemic resistance-related genes (PR1 and PR2) were upregulated and various plant defence activities were detected like the accumulation of ROS, peroxidase and catalase activity. In addition, ZnONPs elicit the levels of SA and ABA phytohormone by increments of 162% and 517% respectively. A similar conclusion was drawn in a study by Singh and colleagues 2018, where it is stated that treatment with the SiO_2NPs and ZnONPs resulted in improved nutrient mobilisation and enhanced soil vigour. Consequently, the overall outcome was improved crop production. According to their findings, 100 μg mL^{-1} concentration of NPs is sufficient to provide resistance to plants and increase agronomic yield.

Das *et al*., 2015 used NP based RNAi approach to silence *AaSNF7* and *AaSRC* through feeding food containing NP-dsRNA by mosquito larva. They synthesised Chitosan NP-dsRNA complex by the procedure described by Zhang *et al*., 2015. The effectiveness of the method was determined by quantitative real-time PCR (qRT-PCR) and the results depicted that was successful activation of RNAi. Feeding larvae with ds*AaSNF7* and ds*AaSRC* conjugated with chitosan NP resulted in 38% and 90% respective decrement in mRNA levels. They experimented using three NPs i.e., chitosan, carbon quantum dot (CQD) and silica. Mosquito genes that were targeted were *SNF7* and *SRC* to control *Aedes aegypti* larvae. To determine the knockdown efficiency of genes by NP-dsRNA complex, relative mRNA levels were assessed.

Studies have shown, the half-life of naked dsRNA is about an hour when maintained in serum (Bartlett *et al*., 2006). It is clear that instability of naked dsRNA on the plant or leaf surface is the limiting factor that shortens the protection window. To overcome this issue, Mitter and his colleagues found out a unique way of delivering dsRNA by loading it on layered double hydroxide (LDH) nanosheets and

spraying it onto the leaves. This approach ensures the sustained and long-lasting effect of dsRNA. These nanosheets are environmentally safe nanoformulation. The ones loaded with dsRNA are termed as 'Bioclay'. When plants are drizzled with bioclay, water vapours present in the environment along with the atmospheric CO2 results in the breakdown of nanosheets and thus allow the slow and continuous release of dsRNA. This provides longer protection period for almost about 30 days after treatment (Mitter *et al.,* 2017).

Tenllado and colleagues 2003, carried out the experiment where they applied naked dsRNA on *N. tabacum* cv. Xanthi leaves. They confirmed resistance against PMMoV up to 5 days after the treatment. Mitter and colleagues 2017, repeated the same experiment but with a little difference. LDH nanosheet loaded with the nucleotide was used in spite of naked dsRNA and it was observed that using bioclay broadens the protection window for about 20 days. The same was also confirmed by applying dsRNA which is homologous to 2b suppressor of *Cucumber mosaic virus* (CMV) on cowpea plants. Mitter *et al*., 2017 also proved that the new leaves were also carrying dsRNA because of systematic movement and were immune to the viral infection.

Konakalla *et al*., 2016, investigated whether TMV infection is affected by dsRNA molecules that is either produced in vitro or applied exogenously. Four simultaneous series of experiments were performed using the following treatments: TMV plus dsRNA_p126, TMV plus dsRNA_CP, TMV (positive control) and water (negative control). Continuous observation was done up to 20 dpi for any expression of infection. Plants treated with TMV/ dsRNA_p126 showed greater resistance i.e. 65% and the ones treated with TMV/dsRNA_CP showed 50% of resistance whereas TMV treated plants showed 100% disease incidence. It is clear from the study that both the treatments are efficient in providing the resistance against TMV infection.

Very recently, Demirer *et al*., 2020, substantiate the delivery of various ss siRNA into intact plant cells of *N. benthamiana* leaves. They intended to knock down the expression of green fluorescent protein transgene in transgenic *mGFP5 N. benthamiana.* As nanocarrier they used single-walled carbon nanotubes (SWNTs). siRNA internalisation was successful within 6 hours of application onto the leaves and consequently, GFP gene was efficiently silenced. Prolonged silencing was observed as π-π adsorption of siRNA delayed intracellular breakdown of siRNA.

Just a while ago, with the similar approach to deliver siRNA, Schwartz *et al*., 2020, exploited additional type of nanoparticles, described as, carbon dots to compose nanoformulation. They experimented on *Nicotiana benthamiana* and tomato plant employing low-pressure spray technique using spreading surfactant that ensured silenced GFP transgenes strongly in both the plant species.

This approach of using nanoparticles loaded with dsRNA has been applied to various insects like aphids (Thairu *et al*., 2017), mosquitoes (Zhang *et al*., 2015; Das *et al*., 2015), and honey bee (Li-Byarlay *et al*., 2013). Hence it provides a potential scope in targeting the viral vectors too.

METHODOLOGY

1. Isolation of viral strain and identitification of disease causing gene:

The plants will be grown under controlled conditions in the polyhouse.

1.1 Collection of virus-infected plant material and genomic DNA extraction- Leaves exhibiting yellow patches will be collected and total genomic DNA will be isolated using CTAB (cetyl trimethyl ammonium bromide) method (Srivastava *et al*.., 1995).

1.2 Rolling circle amplification and cloning of full-length viral genome- Using isolated DNA, viral genome will be amplified using RCA kit as per the instructions given.

1.3 Sequencing and phylogenetic analysis- Sequences will be analyzed using softwares like Molecular Evolutionary Genetics Analysis (MEGA 5.2.2). Comparision for database sequence will be carried out using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). For construction of phylogenetic tree, MEGA v7.0 program will be used (Kumar *et al*., 2017; Agnihotri *et al*., 2019).

2. Preparation of dsRNA (precursor siRNA) constructs: siRNA construct will be prepared refering to one step PCR approach described by Voloudakis *et al*., 2015.

- Mix PCR amplified target DNA with the components given in the replicator RNAi kit in accordance with the instruction given followed by agarose gel electrophoresis.
- dsRNA will be purified by stepwise precipitation with accordance to the instructions given in the kit using lithium chloride solution.
- The obtained pellet will be washed using 70% ethanol.

3. Formulation of Nanoparticles:

3.1 Preparation of cationic nanoparticles- Cationic nanoparticles will be formulated by oil in water emulsion method by following the protocol given by Misra *et al*., 2017 with slight variations. The procedure is as follows:

- PLGA polymer will be dissolved in figured concentration of organic solution containing emulsifing agent.
- Using a microtip probe sonicatorset, prepared emulsion will be sonicated followed by placing it on magnet stirrer.
- Powdered NPs will be obtained using ultra-centrifugation followed by lyophilization.

3.2 Characterisation of prepared nanoparticles- Particle size, zeta potential, surface morphology will be assessed by techniques like dynamic light scattering (DLS) using a zetasizer, atomic force microscopy (AFM) (Misra *et al*., 2017), electron microscopy (Mitter *et al*., 2017).

4. Loading of dsRNA on Nanoparticles:

siRNA will be surface adsorbed following the method given by Misra *et al*., 2017 with slight variations and efficiency of adsorption will be assessed by gel retardation assay. The procedure is as follows:

- To the prepared NPs, siRNA will be added dropwise and will be mixed quickly by gently inverting the tubes.
- At room temperature, mixture will be incubated on rotator followed by lyophilization.
- Lyophilized sample will be dissolved in water and will be analysed by 1% agarose gel electrophoresis.

5. Efficacy analysis of dsRNA loaded NPs against virus:

5.1 Viral load estimation and effect in disease incidence-

qRT-PCR (quantitative real time reverse transcriptase-polymerase chain reaction) will be used to detect mRNA levels in extracts of total plant RNA after the delivery of siRNA loaded NPs (Mitter *et al*., 2003) and also to examine the effect of siRNA on early stage viral infection (Konakalla *et al*., 2016).

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